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Value of Enterobacterial Repetitive Intergenic Consensus PCR for Study of *Pasteurella multocida* Strains Isolated from Mouths of Dogs

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Fifty-six *Pasteurella multocida* strains (40 *P. multocida* subsp. *septica* and 16 *P. multocida* subsp. *multocida* strains) isolated from the mouths of 56 dogs among the 134 living in a French canine military training center (132e Groupe Cynophile de l'Armée de Terre, Suippes, France) were studied by use of enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) and restriction fragment length polymorphism (RFLP) techniques. Both techniques showed genomic heterogeneity of the strains studied. However, RFLP was more discriminatory than ERIC-PCR for differentiating *P. multocida* strains. All but three pairs of strains were discriminated by RFLP, suggesting a limited circulation of strains between these dogs living in proximity. Although ERIC-PCR is easier and faster to perform, it cannot be recommended for epidemiological studies of *P. multocida* strains.

Pasteurella multocida is a commensal of digestive and respiratory tracts of warm-blooded animals and is responsible for diseases chiefly in reared animals (bovine animals, porcine animals, rabbits, and poultry) weakened by stresses such as viral infections, cold, or humidity (5). The bacterium spreads from animal to animal by aerosols. Diseases consist mainly of hemorrhagic septicemia and pneumonia (11). Thus, enzootic pasteurellosis and epizootic pasteurellosis affect the livestock industry and are responsible for important economic losses in cattle farming (10, 29).

Pasteurellosis is a zoonosis, and humans are accidental hosts, acquiring *P. multocida* infections from animals, primarily after cat bites and, to a lesser extent, dog bites (26). The latter infections are, however, more prevalent, as they represent 80% of animal-bite wounds (8). In most cases, infections remain limited to the wound site and have a favorable outcome. However, septic arthritis may occur.

The dogs living in a canine military training center (132e Groupe Cynophile de l'Armée de Terre, Suippes, France [132e GCAT]) are responsible for about 200 bite wounds on dog attendants per year. Most of these wounds have no consequences, as amoxicillin-clavulanic acid is systematically administered. Less than 5% become infected and necessitate hospital consultation. To evaluate the risk of pasteurellosis in the staff of this center, we studied the aerobic bacterial flora of the 134 dogs present in the center in 1996 (17). *P. multocida* was the main species isolated, and 56 strains were recovered from 56 dogs.

Serotyping is commonly used for the epidemiological study of *P. multocida*. Five capsule groups (A, B, D, E, and F) and 16 somatic types (1 through 16) have been described (1, 9, 19). Serotypes of *P. multocida* can be associated with specific diseases in animals (e.g., B:2 and E:2 with hemorrhagic septicemia in cattle and buffaloes) (30). Like other phenotypic markers, the serotype is of limited value for epidemiological studies of *P. multocida* infections (24, 30, 31). To our knowledge, serotyping is no longer used in France and therefore was not used in this study. To characterize the genetic relationship among *P. multocida* strains isolated from the mouths of these dogs living in proximity, we performed enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR). This technique consists of the amplification of genomic DNA enclosed between conserved repetitive regions scattered all over the bacterial genome. These conserved repetitive elements (ERIC sequences) were described first for the genomes of enterobacteria and later for those of many other bacterial species. A consensus sequence has been defined (13, 28). The number and the location of ERIC sequences vary not only between species but also between strains of the same species (13). Electrophoresis of amplified fragments provides band patterns which permit the differentiation of strains. We evaluated this technique for *P. multocida* strains and compared the results to those obtained by use of the restriction fragment length polymorphism (RFLP) technique, a previously validated technique for the epidemiological study of *P. multocida* (24, 27, 30, 31). *P. multocida* is divided into three subspecies (*P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica*, and *P. multocida* subsp. *gallicida*) which have different ecologies (e.g., *P. multocida* subsp. *gallicida* is isolated from avian origins) (5, 12). Thus, the strains of *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica* isolated were studied separately.

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MATERIALS AND METHODS

Dogs. At the time of the study, 134 1- to 11-year-old healthy male dogs had been present at 132e GCAT from 1 month to 8 years. The population consisted of German and Belgian shepherds. The dogs were separated into three groups dwelling in three different enclosures: 109 watch dogs in training, 19 resident dogs belonging to staff, and 6 dogs in specific training (explosives or drugs). Each dog was fed in its own cage.

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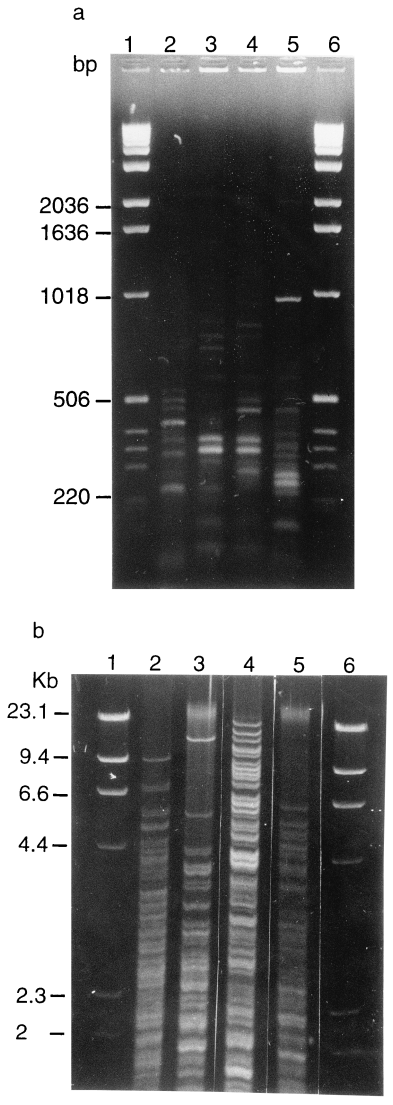

FIG. 1. Genomic fingerprints of *P. multocida* strains obtained by ERIC-PCR (a) and RFLP (b). Lanes 1 and 6, size markers (1-kb DNA ladder and bacteriophage λ DNA digested with *Hin*dIII for ERIC-PCR and RFLP, respectively); lanes 2 to 5, strains of *P. multocida* subsp. *septica* isolated from dogs (strains P30, P31, P32, and P33 respectively).

***P. multocida* strains.** *P. multocida* strains were isolated from dogs by gingival swabbing as previously described (17). Briefly, two cotton-tipped swabs were rubbed vigorously on lateral gums through the muzzle of each dog. The samples were immediately inoculated on sheep blood agar plates and chocolate agar plates and incubated in a 5% CO_2 atmosphere at 37°C. *Pasteurella* strains were identified as described by Holmes et al. (11). Fifty-six *P. multocida* strains were isolated and designated P1 to P56. *P. multocida* subspecies were determined by study of the fermentation of sorbitol and dulcitol. Eight epidemiologically unrelated *P. multocida* strains isolated from eight different patients between 1994 and 1997 at the University Hospital Center of Nancy were also studied (dog-bite wounds, four strains; respiratory tract infections, four strains); they were designated NER1 to NER8 (for "not epidemiologically related" [NER]). *P. multocida* subsp. *multocida* CIP959 (Institut Pasteur, Paris, France) was included as a reference. Ten different colonies were analyzed separately to test the reproducibility of ERIC-PCR and RFLP. Strains were stored at −80°C in brain heart infusion broth with 15% (vol/vol) glycerol.

ERIC-PCR. Bacteria were grown overnight at 37°C in brain heart infusion broth, and suspensions were adjusted to an absorbance at 600 nm of 0.5. Each 25-μl reaction mixture contained 10 μl of bacterial suspension and final amounts of 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 6.7 μM EDTA, 30 mM β-mercaptoethanol, 0.17 mg of bovine serum albumin per ml, 10% (vol/vol) dimethyl sulfoxide, 1.25 mM each deoxynucleoside triphosphate (Boehringer Mannheim Biochemicals, Mannheim, Germany), 2 μM each primer, and 1.5 U of *Taq* DNA polymerase (Gibco-BRL Life Technologies, Paisley, United Kingdom). PCR was performed by use of thermal cycler (GeneAmp PCR System 2400; Perkin-Elmer, Norwalk, Conn.) with an initial denaturation step (95°C, 10 min), with 30 cycles of denaturation (94°C, 1 min), annealing (52°C, 1 min), and extension (65°C, 8 min), and with a final extension (65°C, 16 min). The primers were ERIC 1R (5′-ATG TAA GCT CCT GGG GAT TCA C-3′) and ERIC 2 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′) (28). A negative control without template DNA was included in each run. Amplified products were resolved by electrophoresis in 1.5% (wt/vol) agarose gels containing ethidium bromide (1.6 mg/ml) at 11 V/cm for 90 min in TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA [pH 8]). A 1-kb DNA ladder was used as a size marker (Gibco-BRL Life Technologies).

RFLP. Preliminary studies of restriction endonuclease digestion of *P. multocida* DNA with *Bam*HI, *Bgl*II, *Eco*RI, *Hha*I, *Hin*dIII, *Hpa*II, *Pst*I, and *Sma*I confirmed that *Hha*I gave the most discriminant fingerprint profiles. Therefore, DNA was extracted and digested with *Hha*I (New England BioLabs, Beverly, Mass.) as described by Wilson et al. (30). DNA fragments were separated by electrophoresis in 0.8% (wt/vol) agarose gels containing ethidium bromide (1.6 mg/ml) at 6 V/cm for 7 h in TBE buffer. Bacteriophage λ DNA digested with *Hin*dIII was used as a size marker (Gibco-BRL Life Technologies).

Analysis of band patterns. Amplified products or restriction fragment patterns obtained by ERIC-PCR or RFLP, respectively, were visualized by UV transillumination, and pictures were taken with a charge-coupled device camera (Bio-Rad Laboratories, Richmond, Calif.). Fingerprints were stored in tagged-image-file format with Molecular Analyst 2.1 software (Bio-Rad Laboratories). Images were then processed with Molecular Analyst/PC Fingerprinting software (Bio-Rad Laboratories). Two fingerprints were considered identical if the same numbers of bands at the same positions were observed; variations in intensity were not considered. Percentages of similarity between two profiles were calculated by use of the Dice coefficient × 100 {i.e., $[2n_{AB}/(n_A + n_B)] \times 100$, where n_{AB} is the number of bands common to both strains and $(n_A + n_B)$ is the total number of bands found for both strains} (4). The percentage of similarity ranged from 0% (complete dissimilarity) to 100% (identity). Cluster analysis was performed by use of the unweighted-pair-group method with arithmetic linkages (UPGMA) (14). Strains with a percentage of similarity above 90% were considered only potentially related.

TABLE 1. Comparison of the discriminating values of ERIC-PCR and RFLP

Organism	Strains	Method	Similarity (% range) of:			
			P. multocida subsp. *septica* strains		*P. multocida* subsp. *multocida* strains	
			Dog	NER	Dog	NER
P. multocida subsp. *septica*	Dog	ERIC-PCR	30–100	30–77	23–77	36–72
		RFLP	36–100	36–75	38–86	38–87
	NER	ERIC-PCR		45–82	36–72	36–72
		RFLP		51–57	38–87	51–64
P. multocida subsp. *multocida*	Dog	ERIC-PCR			38–100	38–82
		RFLP			54–100	54–74
	NER	ERIC-PCR				41–79
		RFLP				55–81

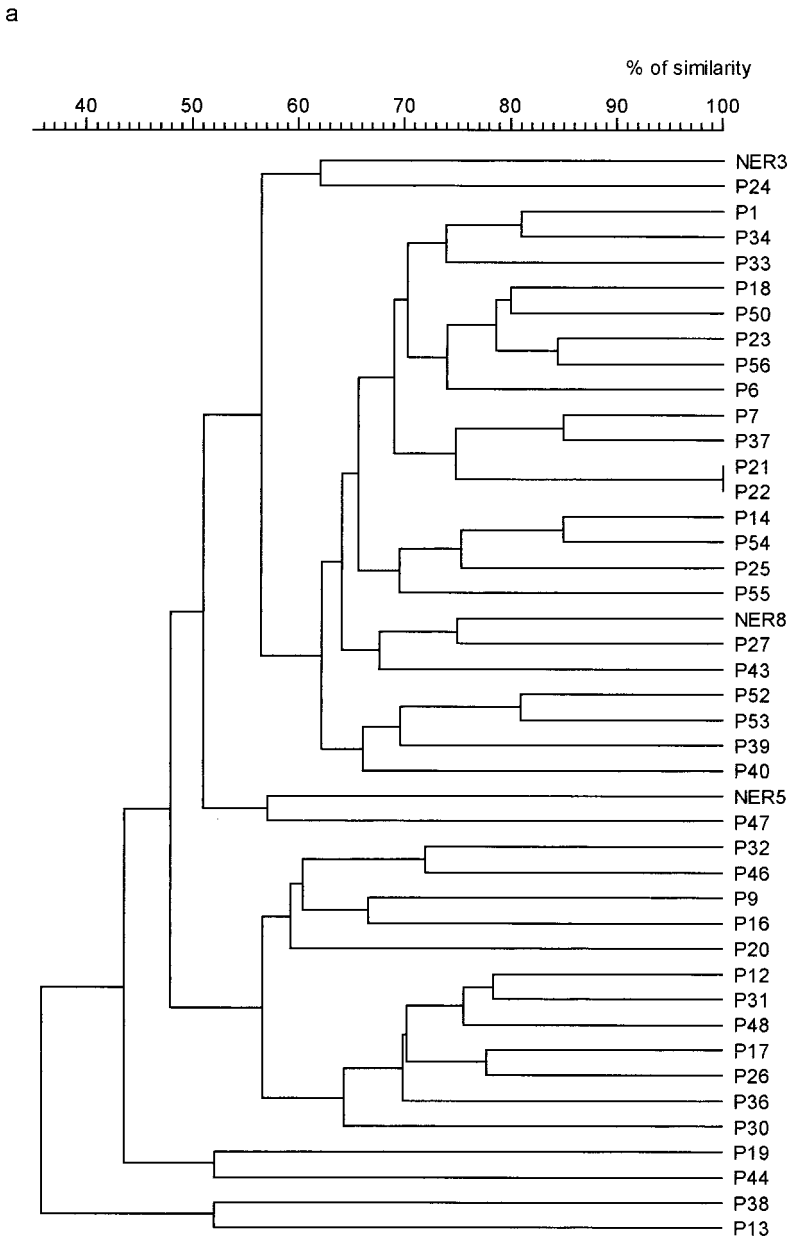

FIG. 2. Dendrograms obtained from RFLP patterns. Percentages of similarity between patterns were calculated by use of the Dice coefficient. Dendrograms were constructed by use of UPGMA. (a) *Pasteurella multocida* subsp. *septica*. (b) *Pasteurella multocida* subsp. *multocida*.

RESULTS

Molecular typing by ERIC-PCR. The DNA of all the isolates of *P. multocida* was amplified by the consensus primers. The fingerprints obtained consisted of 5 to 15 amplification bands ranging in size from 100 bp to 5 kb (Fig. 1a). Sixty-two different patterns were obtained with the 56 dog strains and the 8 NER strains. The 56 dog strains were distributed into 40 *P. multocida* subsp. *septica* and 16 *P. multocida* subsp. *multocida* strains. The NER strains included five *P. multocida* subsp. *multocida* and three *P. multocida* subsp. *septica* strains. The subspecies of *P. multocida* were differentiated by a similarity between their patterns of <78% (Table 1). Among strains of *P. multocida* subsp. *septica*, a similarity of >90% was obtained for two pairs of dog strains: 92% for P6 and P55 and 100% for P21 and P22 (data not shown). Among strains of *P. multocida* subsp. *multocida*, a similarity of >90% was obtained for three pairs of dog strains: 94% for P28 and P41, 96% for P42 and P49, and 100% for P2 and P5 (data not shown). The NER strains were differentiated from each other and from dog strains by a similarity of <83% (Table 1). The 10 patterns obtained with reference strain CIP959 were all identical. They differed clearly from those of dog or NER strains by <64% similarity.

Molecular typing by RFLP. Fingerprints obtained with RFLP consisted of 15 to 35 amplification bands ranging in size from 2 to 23 kb (Fig. 1b). Strains P21 and P22 of *P. multocida* subsp. *septica* showed 100% similarity between their patterns (Fig. 2a). A similarity of >90% was obtained for two pairs of *P. multocida* subsp. *multocida* strains: 98% for P42 and P49 and

b

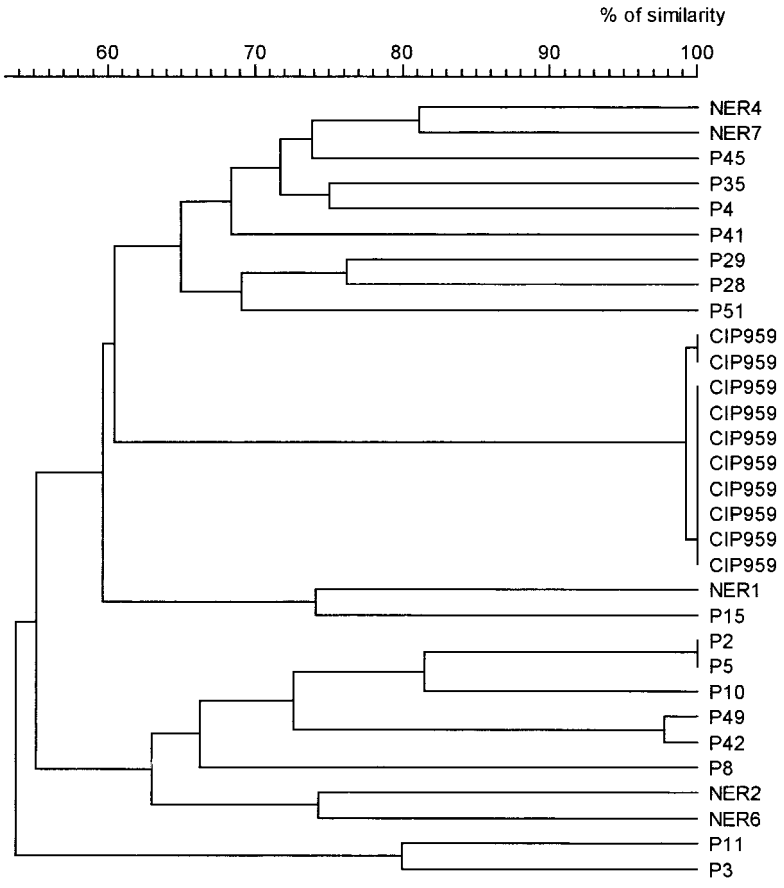

FIG. 2—*Continued.*

100% for P2 and P5 (Fig. 2b). The NER strains were differentiated from each other and from dog strains by a similarity of <88% (Table 1). Reference strain CIP959 gave eight identical fingerprints and two patterns showing 99% similarity with the other eight patterns. These 10 patterns differed from those of dog or NER strains by a similarity of <61% (Fig. 2b).

DISCUSSION

Two molecular epidemiological techniques have been recently applied to *P. multocida*: random amplified polymorphic DNA (2, 22) and RFLP (24, 27, 30, 31). However, random amplified polymorphic DNA, which gives less than six amplification bands, is less discriminatory than RFLP, which yields patterns consisting of more than 10 restriction fragments. RFLP has been shown to be of value for the differentiation of field and vaccine isolates (30, 31) and for evidencing the probable source of infection in a patient who died of endocarditis due to *P. multocida* (27).

ERIC-PCR, a recently described epidemiological technique (28), has been previously used for various species belonging to α-proteobacteria (21), γ-proteobacteria (3, 7, 15, 16, 18, 20, 28), and ε-proteobacteria (6). ERIC sequences have also been described for the genomes of *Staphylococcus aureus* (25) and *Mycobacterium tuberculosis* (23). As ERIC sequences are present in most γ-proteobacteria, it is not surprising that they are also present in the genome of *P. multocida*.

P. multocida subsp. *septica* and *P. multocida* subsp. *multocida* were differentiated by a similarity of <78% with ERIC-PCR. For these subspecies, differentiation between dog strains and NER strains was satisfactory, as the similarity between strains belonging to these different groups was never >82%. Good reproducibility of this technique was also observed, as the 10 patterns obtained with the reference strain were all identical. However, differentiation between strains belonging to the same subspecies was less satisfactory. Five pairs of strains showed >90% similarity in ERIC-PCR. This apparent relation was not confirmed by RFLP for two of these pairs, as strains P6 and P55 and strains P28 and P41 showed 65% similarity between their patterns, clearly differentiating them. The three other pairs (P42 and P49; P2 and P5; and P21 and P22) had closely related patterns in ERIC-PCR (96, 100, and 100% similarity, respectively) and in RFLP (98, 100, and 100% similarity, respectively). Among these five pairs of strains, only strains P42 and P49, strains P2 and P5, and strains P21 and P22, which had similar or identical patterns with both techniques, were epidemiologically related. They were isolated from resident dogs (P42 and P49; and P21 and P22) which dwell in the same enclosure or from watch dogs in training (P2

and P5). Thus, they may be considered as belonging to the same clones. This information indicates that at 132e GCAT, transmission between animals is a possibility. These dogs do not share food or water; however, transmission could take place during training (e.g., biting of the same stick) or by aerosol transmission between dogs dwelling in the same enclosure.

In a preliminary study, gingival swabbing of 25 dogs at 132e GCAT was performed. Isolation plates for samples from 10 of these dogs yielded three to five colonies of *P. multocida* per dog. The corresponding 39 strains were studied by RFLP. All the strains from each dog had the same profile, suggesting that a dog may harbor only one strain of *P. multocida*. All 10 profiles were different (unpublished results). Thus, a dog may retain the *P. multocida* strain that it harbored when entering the training center. This suggestion would explain the clonal diversity of *P. multocida* strains in a situation in which transmission between dogs would be limited. If few strains had circulated at 132e GCAT, a higher prevalence of identical or closely related patterns would have been obtained. However, it would have been necessary to collect iterative samples from the same dogs to study the evolution of *P. multocida* colonization of the mouths of the dogs.

ERIC-PCR is a valuable technique for epidemiological studies. It provides reproducible results and has satisfactory discriminatory power, as all NER strains and most dog strains were differentiated by this technique. However, the thresholds of identity (96%) and difference (94%) between strains are very close, impairing the interpretation of results. Such limited discrimination has also been reported for *Enterobacter aerogenes* (7). ERIC-PCR is easier and faster to perform than RFLP; however, it cannot be recommended at the present time for epidemiological investigations of *P. multocida* strains. Further studies are necessary for establishing its usefulness as a screening tool.

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